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The Zta Transactivator Involved in Induction of Lytic Cycle Gene Expression in Epstein-Barr Virus-Infected Lymphocytes Binds to Both AP-1 and ZRE Sites in Target Promoter and Enhancer Regions

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The BZLF1 or *zta* immediate-early gene of Epstein-Barr virus (EBV) encodes a 33-kilodalton phosphorylated nuclear protein that is a specific transcriptional activator of the EBV lytic cycle when introduced into latently infected B lymphocytes. We have shown previously that the divergent EBV DSL target promoter contains two *zta*-response regions, one within the minimal promoter and the other in an upstream lymphocyte-dependent enhancer region. In this study, we used footprinting and gel mobility retardation assays to reveal that bacterially synthesized Zta fusion proteins bound directly to six TGTGCAA-like motifs within DSL. Four of the Zta-binding sites lay adjacent to cellular TATA and CAAT factor-binding sites within the minimal promoter, and two mapped within the enhancer region. Single-copy oligonucleotides containing these Zta-binding sites conferred Zta responsiveness to heterologous promoters. In addition, the Zta protein, which possesses a similar basic domain to the conserved DNA-binding region of the c-Fos, c-Jun, GCN4, and CREB protein family, proved to bind directly to the consensus AP-1 site in the collagenase 12-*O*-tetradecanoylphorbol-13-acetate response element. Cotransfection with *zta* also *trans* activated a target reporter gene containing inserted wild-type 12-*O*-tetradecanoylphorbol-13-acetate response element oligonucleotides. Cellular AP-1 binding activity proved to be low in latently EBV-infected Raji cells but was induced (together with the Zta protein) after activation of the lytic cycle with 12-*O*-tetradecanoylphorbol-13-acetate. We conclude that EBV may have captured and modified a cellular gene encoding a c-*jun*-like DNA-binding protein during its evolutionary divergence from other herpesviruses and that this protein is used to specifically redirect transcriptional activity toward expression of EBV lytic-cycle genes in infected cells.

The ability of immediate-early nuclear regulatory proteins of DNA viruses to initiate temporal and tissue-specific expression of appropriate target genes in infected cells requires a complex interplay with cellular transcription factors. In the case of Epstein-Barr virus (EBV), three phosphorylated nuclear transactivator proteins have been described that either specifically or nonspecifically stimulate gene expression from lytic-cycle viral promoters in transient cotransfection assays (10, 26, 43, 64). The lytic cycle of EBV can be reactivated from latently infected B lymphocytes in culture after various chemical manipulations, including treatment with tumor promoters (68), sodium butyrate, anti-immunoglobulin M (IgM), and calcium ionophores as well as by superinfection with rearranged defective P3HR-1 virus genomes in which all three EBV transactivators potentially come under the transcriptional control of the *Bam*HI-W or -C latency promoters (12, 45).

One of the transactivator genes of EBV, referred to as BZLF1, Z, EB1, or *zta*, encodes a 33-kilodalton (kDa) nuclear protein and is an immediate-early gene that is transcribed in the presence of cycloheximide in EBV-positive cells after induction with anti-IgM antibodies (63). This gene product (but neither of the other two EBV lytic-cycle transactivators) is sufficient on its own to reactivate the entire lytic cycle process when introduced into EBV latently infected lymphoblastoid cells (14, 15). In transient cotransfection assays, the Zta gene product, when expressed from a simian virus 40- or retrovirus long terminal repeat-driven effector plasmid, specifically stimulates transcription of several EBV lytic-cycle promoters (8, 10, 26, 32, 64). One particularly responsive target is the divergent promoter in the left-hand side duplicated sequence (DSL), which is activated in both directions (26, 42). The leftward transcript, referred to as the *Not*I repeat transcript (*ntr*), is also known to be highly inducible in 12-*O*-tetradecanoylphorbol-13-acetate (TPA)-treated, latently infected B cells (21, 30). The product of the rightward transcript is partially homologous to the *bcl*-2 cellular oncogene (13, 52). More recently, DSL and its duplicated homolog on the right-hand side of the genome DSR (38) have also been identified as essential components of the EBV lytic origin of DNA replication (25).

To understand the role of the Zta protein in lytic-cycle activation, we dissected the 1,004-base-pair (bp) DSL upstream noncoding region and found two distinct *zta*-responsive domains with different cell-type dependencies (26, 42). The 200-bp leftward minimal promoter region responds almost as efficiently as the wild-type promoter in Vero cells but is activated only weakly in lymphocytes. On the other

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hand, an isolated 260-bp far-upstream domain acts as an inducible enhancer that responds to the *zta* transactivator only in latently EBV-infected IB4 or Raji cells (8, 15, 42). In this paper, we demonstrate that the Zta protein itself is a sequence-specific DNA-binding factor that recognizes multiple target sites within both the minimal and far-upstream response regions. Furthermore, the range of binding sites recognized by Zta includes a TPA-responsive AP-1 site (TRE) from the promoter for the mouse collagenase gene, which has been shown to be a target for binding and transcriptional activation by the products of the c-*jun* and c-*fos* proto-oncogenes (16).

Recently, Farrell et al. (19) suggested that the Zta (BZLF1) protein has homology to the c-Jun–c-Fos family of cellular transcription factors and also presented evidence showing that bacterially synthesized Zta protein binds directly to an AP-1 consensus site (TGAGTCA) in the EBV BMLF1 promoter. However, they provided no evaluation of whether this is a functionally significant site, and they did not examine the target site binding specificity.

MATERIALS AND METHODS

Recombinant plasmids and synthetic oligonucleotides. The SV-Z effector plasmid pPL17 contains the EBV *Bam*HI-Z fragment inserted in place of the *neo* gene in SV2-neo and has been described previously (26). Plasmids pSV-cFos, pSV-cJun, and pSV-JunB, containing the cDNAs for c-Fos, c-Jun, and JunB, were obtained from Kevin Ryder, Laura Saunders, and Daniel Nathans, Johns Hopkins University School of Medicine. Target plasmids TRE/TK-CAT and TRE-Δ72/TK-CAT (3) were obtained from Michael Karin, University of California, San Diego. The EBV-derived target genes are shown in Fig. 2a. NRP(−1020/+40)-CAT (pDH123) contains the complete upstream sequences of the *Not*I repeat promoter (NRP) region linked to the chloramphenicol acetyltransferase (CAT) gene (26, 43), whereas MIN(L)-CAT (pPL55B) contains the minimal NRP sequences (−155/+40) required for Vero cell response (42). The construction ENH/A10-CAT (pMH103) contains the B-cell-responsive region from bp-643 to -900 of the NRP promoter inserted at the *Bgl*II site of pA10-CAT (15, 42).

The synthetic oligonucleotides used in binding studies (usually 30-mers with *Bgl*II and *Bam*HI linker sequences) included the following (drawn as the top strand only in 5′-to-3′ orientation): GATCTTCTAGACCAAATGTGCAAAGG TGAG (ZRE5), GATCTTCTAGACCAAATGGTCAAAGG TGAG (ΔZRE5), GATCTGAATTAACCAATAAGAAGCC CCCAG (CAAT), GATCCCATTGACGTCAATGGGGTC AGCTGA (CRE), GATCCTGCCCCTGACTCAGGAGACC ATGGA (RR2), CTTGGTTAATTCAGGTGTGTCATTTTA GATC (TGTG1), GATCTGGGGCTTAGTGTGTCATGGTG AGGCAG (TGTG3). The CRE oligonucleotide contains a proven CREB-binding cyclic AMP-responsive signal derived from the IE94 promoter of simian cytomegalovirus (7). The RR2 control oligonucleotide contains an AP-1-like sequence derived from the promoter for the ribonucleotide reductase A subunit gene from herpes simplex virus type 2. The TRE and ΔTRE probes used in the binding studies represented 18-bp *Hin*dIII-to-*Bam*HI fragments isolated from the 5×TRE/TK-CAT and TREΔ72/TK-CAT plasmids (3) and have the following double-stranded sequences: 5′-AGCTTG ATGAGTCAGCCG-3′/5′-GATCCGGCTGACTCATCA-3′ (TRE) and 5′-AGCTTGTGGAGTCAGCCG-3′/5′-GATCCG GCTGACTCCACA-3′ (ΔTRE).

For demonstration of their *zta* response properties, the ZRE5 and ΔZRE5 oligonucleotide pairs were cloned through their *Bam*HI and *Bgl*II termini into the *Bam*HI site at bp-119 in a herpes simplex virus type 1 thymidine kinase (TK-CAT) reporter gene in the plasmid pLS0 to create pPL108a (ZRE5, forward orientation) and pPL109a (ΔZRE5, forward orientation). Similarly, the TGTG3 oligonucleotide pair (which encompasses ZRE6) was cloned into the 5′-upstream *Bgl*II site in pA10-CAT to create plasmids pMH116 (forward orientation) and pMH117 (backward orientation). Finally, the ZRE3 (TGTG1) oligonucleotide pair was inserted as a forward-oriented, tandemly repeated dimer (pPL107) between the *Hin*dIII and *Bam*HI sites in place of the 5×TRE sequence in TRE/TK-CAT.

Transfections, CAT assays, and RNA analysis. Monolayer cultures of 5×10^5 Vero cells were transfected by calcium phosphate precipitation as previously described (43). Suspension cultures of 10^6 IB4 lymphoblastoid cells (33) were transfected by the DEAE-dextran method of Takada et al. (64). Each transfection mixture contained 2 μg of target DNA and 2 μg of effector DNA. Cells were harvested for CAT assays or for RNA analysis 48 h after transfection. CAT assays were performed as described by Lieberman et al. (43). Total cellular RNA from transfected Vero cells was isolated by the guanidinium hydrochloride-cesium chloride procedure and hybridized to an excess of a double-stranded DNA fragment probe generated by *Nco*I digestion of *Bam*HI-H plasmid DNA. The isolated DNA fragment was ^{32}P end labeled with T4 polynucleotide kinase; after S1 endonuclease digestion, the resulting hybrids were analyzed on a 7% acrylamide–8 M urea sequencing gel.

Raji cell nuclear extracts. Raji cell nuclear extracts were prepared by the high-salt extraction procedure described by Dignam et al. (17). The cells were lysed by Dounce homogenization, and the pelleted nuclei were suspended at 3×10^8 cells per ml in buffer containing protease inhibitors (0.5 mM phenylmethylsulfonyl fluoride, 0.1 mM tolylsulfonyl phenylalanyl chloromethyl ketone, and 0.1 mM tolylsulfonyl lysyl chloromethyl ketone). Lytic-cycle induction was carried out by treatment with 40 ng of TPA per ml and 3 mM sodium butyrate for 48 h and confirmed by detection of the EBV EA(D) nuclear antigen (BMRF1 gene product) in 10% of the cells by indirect immunofluorescence with the R3 monoclonal antibody.

Isolation of Zta proteins expressed in *Escherichia coli*. Initially, a nonspliced genomic BZLF1 expression plasmid (pPL38) was constructed by ligating a *Nae*I-to-*Pst*I fragment from *Bam*HI-Z between *Sma*I and *Pst*I in the polylinker sequences of the λN expression plasmid pHE6 (46). The 30-kDa HE-BZLF1 protein contains the first 200 amino acids of the BZLF1 open reading frame fused to 33 amino acids of λN protein and 7 amino acids encoded by the Zta leader sequences (Fig. 1c). Subsequently, an oligo(dT)-primed λgt10 cDNA library derived from TPA-induced B95-8 cells was screened by hybridization with a DNA probe from the 5′ end of the coding region for the *zta* gene. DNA from positive plaques was purified, and the *zta* sequences bounded by *Eco*RI linkers were moved into the Bluescript vector (Stratagene). DNA sequencing revealed that clone pPL102 was intact up to position +31 in the leader sequences of the *zta* gene and extended to the poly(A) signal at the 3′ terminus of the gene (Fig. 1d). This sequence was subsequently transferred as an *Eco*RI fragment into the *Bam*HI site of the *Staphylococcus* protein A expression vector pRIT2T (Pharmacia Fine Chemicals) to form plasmid pPL104. The 60-kDa ProtA-Zta fusion protein expressed from this construction contains 251 amino acids of protein A, followed by 8 amino

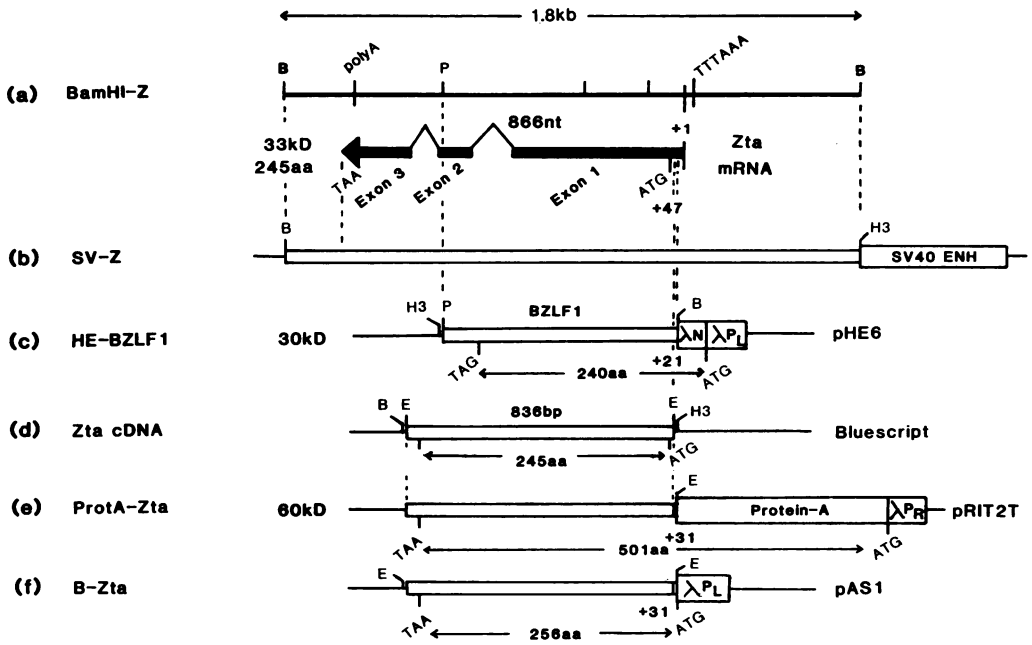

FIG. 1. Structure of the EBV *zta* transactivator gene and the various Zta expression vectors. (a) *Bam*HI-Z: location of the 866-nucleotide spliced *zta* mRNA (spanning positions 101,947 to 103,741) within the parent *Bam*HI-Z DNA fragment of the EBV (B95-8) genome. Restriction sites: B, *Bam*HI; E, *Eco*RI; H3, *Hin*dIII; P, *Pst*I; TTTAAA, consensus TATA-element. (b) SV-Z: diagramatic representation of a portion of the mammalian expression vector plasmid pPL17, which contains the SV-Z effector gene. (c) HE-BZLF1: the genomic region from *Nae*I to *Pst*I of *Bam*HI-Z was introduced into an *E. coli* λP_L expression vector to produce the λN-BZLF1 fusion protein encoded by plasmid pPL38. (d) Zta cDNA: structure of the isolated intact *zta* cDNA clone derived from TPA-induced B95-8 cells. The 836-bp *zta* insert was moved from a phage λgt10 library clone into the Bluescript vector (pPL102). (e) ProtA-Zta: the 836-bp cDNA was inserted in phase into the *Bam*HI site in the protein A expression vector (pRIT2T) to produce a 60-kDa fusion protein containing all 245 amino acids of Zta (plasmid pPL104). (f) B-Zta: insertion of the 836-bp *zta* cDNA in phase into the *Bam*HI site of the λP_L vector pAS1. This construction produces a 34-kDa protein containing all 245 amino acids of Zta plus 11 additional amino acids derived from linker sequences plus the leader region at the 5′ end of the *zta* cDNA. kb, Kilobases.

acids encoded by the Zta leader sequences (SIPLSLLK) plus the complete 245 amino acids of the Zta protein (Fig. 1e). After a 2-h induction at 42°C, the soluble bacterial fusion protein was isolated by a freeze-thaw, high-salt extraction procedure (46, 55) and passed through an IgG-Sepharose column (Pharmacia). Bound material was eluted with ammonium acetate (pH 3.4) and then dialyzed immediately against 20 mM HEPES (*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid) (pH 7.4)–50 mM KCl–12 mM $MgCl_2$–2 mM EDTA–1 mM dithiothreitol–1 mM phenylmethylsulfonyl fluoride–20% glycerol.

The much smaller B-Zta protein was produced by inserting the *zta* cDNA as an *Eco*RV-to-*Xba*I fragment by blunt-end ligation at the *Bam*HI site in the λP_L expression vector pAS1 (20). This construction (pPL122) produces a 34-kDa product with 11 additional amino acids at the amino terminus, which are derived from both linker sequences and the *zta* leader region (Fig. 1f). A soluble high-salt extract was fractionated on a heparin-agarose column, and the B-Zta protein was eluted in buffered 0.5 M NaCl.

Antipeptide antiserum and immunoblotting. A Zta-specific antiserum was raised against a synthetic peptide corresponding to codons 106 to 120 of the predicted Zta amino acid sequence (5). Serine-cysteine was added at the carboxyl terminus of the peptide to provide a spacer and a sulfhydryl group, and D-tyrosine was added to the amino terminus. The peptide was linked to keyhole limpet hemocyanin and used as an immunogen in rabbits. For immunoblotting, 20-μl samples of the Raji cell or bacterial extracts were separated by gel electrophoresis through a 12.5% polyacrylamide gel and transferred electrophoretically to nitrocellulose. The preincubated filters were treated for 90 min at 23°C with a 1:100 dilution of the Zta-specific anti-peptide serum followed by ^{125}I-labeled protein A. For reaction with DNA probes, the proteins on the filter were first renatured by treatment with buffered 6 M guanidinium hydrochloride for 30 min, followed by incubation in 100 mM guanidinium hydrochloride for 6 h at 23°C (23, 47).

DNA-binding and footprinting assays. Typical binding reactions were performed at 4°C for 20 min in 25 mM HEPES (pH 7.9)–70 mM KCl–0.5 mM EDTA–1 mM dithiothreitol–0.5% Nonidet P-40–5% glycerol–20 μg of poly(dI-dC) per ml plus Raji cell nuclear extract, ProtA-Zta, or B-Zta in a final volume of 25 μl (56). Synthetic oligonucleotide probes were end labeled with T4 polynucleotide kinase. DNA restriction fragment probes were 3′ end labeled by filling in with Klenow polymerase and purified by isolation from polyacrylamide gels. Gel mobility retardation assays were carried out by electrophoresis through 6% polyacrylamide gels in 0.03 M Tris–0.03 M boric acid–0.7 mM EDTA (pH 8.0). For footprinting assays, 100-μl binding reactions were incubated at 23°C for 20 min and then treated with 5 ng of DNase I (Worthington Diagnostics) per ml–10 mM $MgCl_2$–1 mM $CaCl_2$ for 30 s, followed by quenching in 100 mM NaCl–50 mM EDTA. After phenol extraction and ethanol precipitation, the DNA was suspended in formamide-dye and subjected to electrophoresis on 7% polyacrylamide–8 M urea sequencing gels.

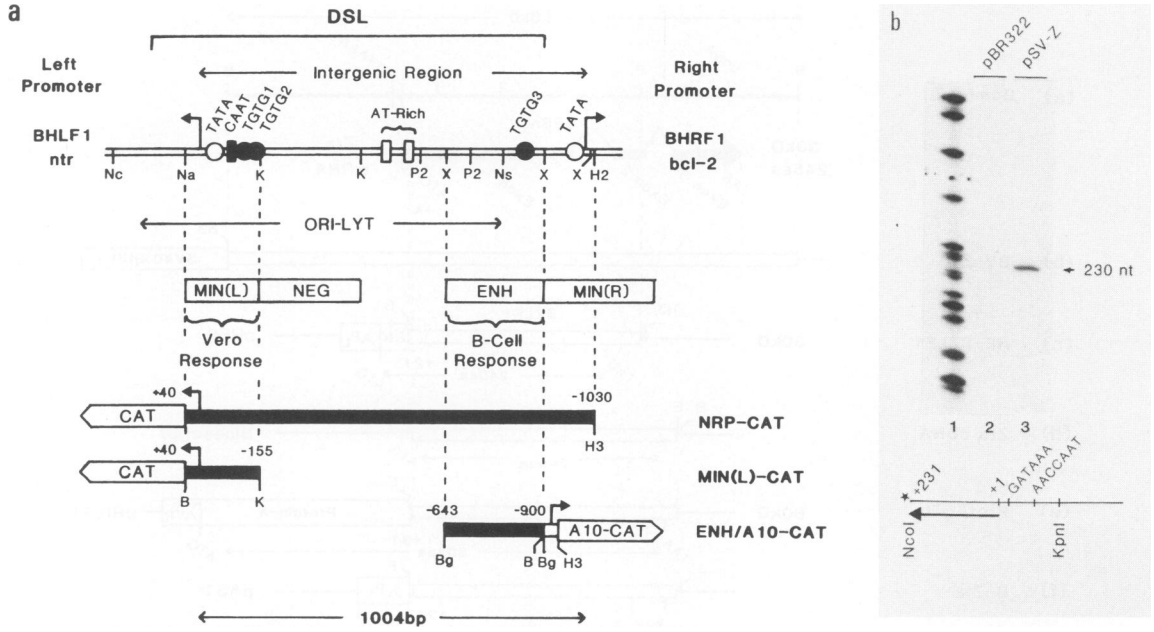

FIG. 2. Organization of the Zta-responsive DSL divergent promoter region and structure of reporter plasmids. (a) Features of the intergenic region within the *Bam*HI-H fragment and the location of relevant portions of three target CAT plasmid constructions used in this work. The 1,004-bp region between the arrows contains nucleotides 52,786 to 53,790 from the 172,000-bp EBV (B95-8) genome. The arrows represent the leftward mRNA start site for the *Not*I repeat (*ntr*) gene (BHLF1 open reading frame) and the rightward mRNA start site for the *bcl*-2 related gene (BHRF1 open reading frame). The entire region designated DSL is also duplicated near the right end of the genome in many strains of EBV. The patterns of *zta* responses for both leftward and rightward transcription (15, 26, 42) define minimal promoter regions, MIN(L) and MIN(R), plus a negative region (NEG) and an upstream enhancer region (ENH). The outer boundaries of the lytic origin of replication (*ori*-Lyt) are indicated (25). The positions of consensus TATA elements (○), a CAAT factor-binding site (■), the three copies of the GGTGTGTCA consensus element (●) and the two 18-bp A+T-rich palindromes (□) are indicated. Restriction sites: B, *Bam*HI; Bg, *Bgl*II; H3, *Hin*dIII, H2, *Hin*cII; K, *Kpn*I; Na, *Nae*I; Nc, *Nco*I; Ns, *Nsi*I; P2, *Pvu*II; X, *Sma*I/*Xma*I. (b) Evidence that *zta*-stimulated expression of *ntr* mRNA from the leftwards DSL promoter occurs by an increase in the level of correctly initiated steady-state mRNA. The figure shows the result of S1 nuclease analysis after annealing of a *Nco*I-to-*Sma*I DNA probe to total RNA (20 μg) extracted from Vero cells transfected with target genomic *Bam*HI-H DNA (pSL77) and either pBR322 DNA (lane 2) or the SV-Z effector plasmid DNA (lane 3). The 870-bp probe DNA fragment was 5′ end labelled at a *Nco*I site at position +231. Lane 1, DNA size markers.

RESULTS

Zta stimulates correctly initiated transcription from the *ntr* gene promoter. The relative organization of the *zta*-responsive regions and the structures of the appropriate reporter plasmids used are summarized in Fig. 2a. To confirm the validity of the *zta* transactivation process in transient assays, we cotransfected an SV-Z effector plasmid (pPL17; Fig. 1b) with the EBV *Bam*HI-H fragment containing the intact viral BHLF1 (*ntr*) target gene driven by the leftward DSL promoter. An abundant *ntr* gene transcript was produced that gave a 230-nucleotide fragment in an S1 protection assay (Fig. 2b, lane 3). In contrast, cotransfection of the same target gene with control pBR322 DNA gave no specific RNA species that was protected from S1 digestion (Fig. 2b, lane 2). The initiation of the *ntr* transcript induced by cotransfection with *zta* correlates precisely with the mRNA start site detected previously for both the endogenous *ntr* transcript in TPA-induced EBV lymphoblastoid cells (30, 38) and for the NRP-CAT reporter gene in transient assays (42).

A bacterially produced Zta fusion protein binds directly to DSL consensus sequences. To determine whether Zta might be a direct DNA-binding protein, we chose to overexpress the *zta* gene as a fusion protein in *E. coli*, a technique that we had used successfully previously for the EBV latent state DNA-binding protein EBNA-1 (55). Initially, a genomic clone containing the complete BZLF1 open reading frame was expressed as a 30-kDa λN fusion protein in *E. coli* (Fig. 1c). This product was recognized by our anti-Zta antibody on Western blots (immunoblots) but was not found to have DNA-binding activity (data not shown). Subsequently, Biggin et al. (5) reported that the immediate-early Z protein was encoded by a spliced viral mRNA consisting of a large 5′ exon from BZLF1 and two small adjacent 3′ exons. Therefore, we isolated a nearly full-length *zta* cDNA clone from a λgt10 library generated from TPA-treated B95-8 cells (Fig. 1d). This 836-bp cDNA was moved into a protein A plasmid vector (pRIT2T) and expressed as an in-phase fusion protein (Fig. 1e). Note that the 5′ end of the cDNA begins at position +31 and includes the natural initiator ATG codon at position +47. Therefore the predicted 60-kDa polypeptide product should contain all 245 amino acids of the Zta protein linked to a 255-amino-acid NH_2-terminal protein A domain. Western blotting analysis confirmed the presence of an appropriately sized fusion protein in the crude bacterial extract, although considerable degradation was also apparent (Fig. 3a).

The sequence motif GGTGTGTCA is repeated twice within the leftward NRP minimal promoter and once as AG TGTGTCA in the enhancer region of the DSL (Fig. 2a, TG TG elements). Therefore, we initially chose to test these sequences as potential targets for recognition by the Zta protein. The crude Zta fusion-protein extract produced specific DNA-protein complexes in a gel mobility retardation assay with labeled 30-mer double-stranded oligonucleotides

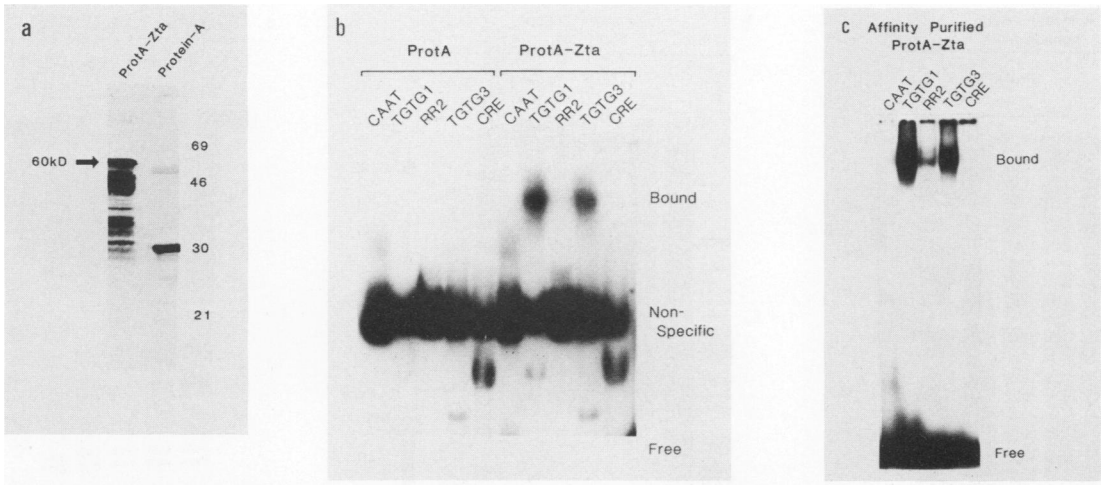

FIG. 3. Synthesis and DNA-binding properties of a bacterially expressed ProtA-Zta fusion protein. (a) Detection of the 60-kDa ProtA-Zta fusion protein by Western immunoblot analysis. Unfractionated supernatant extracts from lysed, heat-induced *E. coli* containing the pRIT2T (ProtA) and pPL104 (ProtA-Zta) plasmids were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis and electroblotted onto nitrocellulose. Protein A-containing products were detected indirectly with rabbit preimmune antiserum, followed by horseradish peroxidase conjugated goat anti-rabbit IgG antibody. (b) Gel mobility retardation assay with various ^{32}P-labeled 30-bp oligonucleotide probes using crude bacterial extracts (ProtA, ProtA-Zta). The band in the center of the gels is a nonspecific binding activity. Free unbound oligonucleotides ran off the bottom of the gel. (c) Similar gel retardation assays with IgG column affinity-purified ProtA-Zta fusion protein.

representing either the leftward or enhancer versions of the repeated TGTG1 and TGTG3 motifs (Fig. 3b). No such complexes were detected with the control unmodified protein A bacterial extracts or with canonical CTF (AACCAAT) or CRE (TGACGTCA) control oligonucleotides. When the ProtA-Zta fusion protein was partially purified over an IgG affinity column, TGTG1- and TGTG3-specific DNA-binding activity was recovered free from nonspecific bacterial DNA-binding proteins (Fig. 3c).

Additional Zta-binding sites revealed by DNase I footprinting. To examine the possibility that other specific binding sites for the Zta fusion protein might occur within the DSL *zta*-responsive regions, we carried out footprinting experiments with the IgG affinity-purified material. The bacterial Zta protein proved to protect a 20-bp region centered over the AGTGTGTCA sequence within the 260-bp enhancer fragment (Fig. 4a), as was predicted from its ability to bind to the appropriate oligonucleotide (TGTG3). However, the purified Zta fusion protein also had an approximately four-fold higher affinity for a previously unrecognized site within the DSL enhancer response region (Fig. 4b). This higher-affinity site lies within a 16-bp protected region centered over the core sequence TGTGCAA. These two ProtA-Zta-binding sites will subsequently be referred to as ZRE6 and ZRE5, respectively (see Fig. 10).

We next examined the interactions of the affinity-purified Zta protein with the minimal leftward promoter sequences in the Vero cell response region. From the oligomer gel retardation binding assays, we anticipated that the Zta protein would bind to the duplicated GGTGTGTCA elements at positions −110 and −140. However, we found instead that it protected a large 34-bp region at positions −75 through −41 between the CAAT element and the TATA motif (Fig. 4c). The binding affinity for these sequences was nearly 1 order of magnitude greater than that for the TGTG1 and TGTG2 elements, which were only minimally protected. The high-affinity site within the minimal promoter region appeared likely to encompass two adjacent related motifs (TGTGTAA at position −50 and TGAGCAA at position −68), which are each similar to the ZRE5 core sequence from the enhancer region (TGTGCAA) and are arranged here in a head-to-head configuration.

Further resolution of the promoter-proximal Zta-binding sites with a bacterial nonfusion Zta protein. To produce a more authentic version of the Zta protein without the bulky protein A fusion domain we also placed the cDNA into the *E. coli* pAS1 vector (Fig. 1f). The B-Zta protein was identified by Western immunoblotting with a polyclonal rabbit antiserum generated against codons 106 to 120 of the predicted EBV Zta polypeptide. This antiserum detects the induction of a nuclear Zta antigen in latently EBV-infected Raji cells only after TPA treatment (15). All protein A-derived polypeptides (with or without Zta-derived domains) were expected to be detected in the ProtA-Zta extract, but only Zta-containing polypeptides should be detected in the nonfusion B-Zta extract (Fig. 5a). The partially purified bacterial B-Zta protein obtained after heparin-agarose fractionation proved to be of almost exactly the same size as the authentic 33-kDa mammalian cell-derived EBV Zta protein that was present in unfractionated nuclear extracts of TPA-treated Raji cells. Furthermore, despite the presence of multiple protein A degradation products that were detected by immunoblotting, only an intact 60-kDa form and a minor 45-kDa form of ProtA-Zta were detected by Southwestern blotting to the IgG affinity-purified sample with a labeled high-affinity ZRE5-binding site oligonucleotide probe (Fig. 5b). The specificity of this latter interaction was confirmed by the lack of binding to a point mutant version of the oligonucleotide (ΔZRE5).

The 256-amino-acid B-Zta protein was used to further examine the interactions with the minimal promoter sequences. DNase I footprinting analysis confirmed that the large protected region previously observed with ProtA-Zta contained two distinct Zta-binding sites with an intervening hypersensitive site at positions −60 and −61 (Fig. 6). These two binding sites will be referred to as the TATAA-proximal ZRE1 locus at positions −42 to −59 and the CAAT-proximal ZRE2 locus at positions −62 to −77. Furthermore, in this experiment full protection occurred at positions −98 to −115 and −132 to −148, corresponding to the two GGTGTGTCA

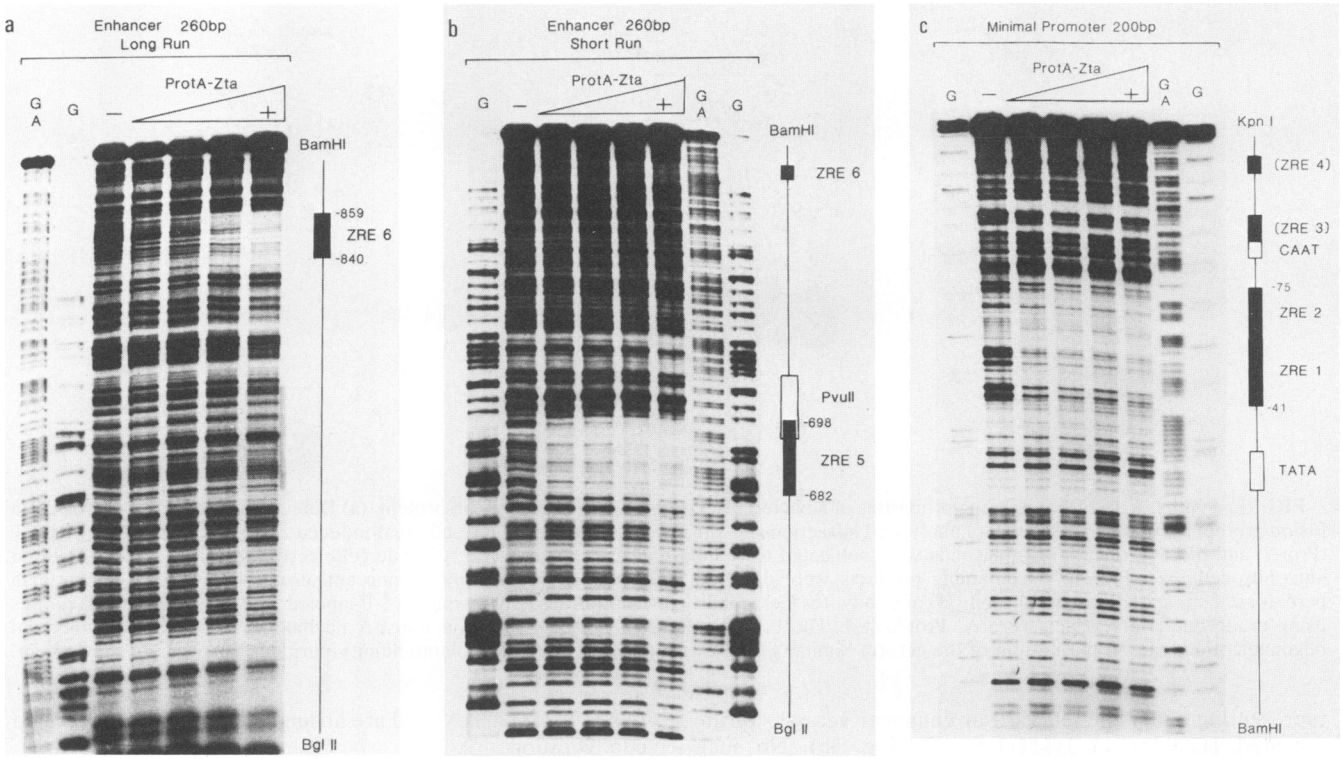

FIG. 4. Footprinting analysis of ProtA-Zta-binding sites in the DSL enhancer and minimal promoter regions. The autoradiographs show partial DNase I digestion patterns of ^{32}P-end-labeled DNA fragments in the presence or absence of increasing amounts of the ProtA-Zta fusion protein. Control chemical sequencing lanes (G or G plus A) with the same DNA samples were used for reference. (a and b) Long and short electrophoresis runs, respectively, with the lymphocyte enhancer region. The 260-bp *Xma*I-to-*Xma*I fragment was isolated by using the polylinker *Bgl*II and *Bam*HI sites in the ENH/A10-CAT plasmid pMH103 and 5′ end labeled at the *Bgl*II site. Lanes: −, no extract; +, incubation with 1, 2.5, 5, and 10 μl of IgG column affinity-purified ProtA-Zta fusion protein. (c) Analysis of the proximal response region. The 200-bp *Bam*HI-to-*Kpn*I fragment was isolated from the MIN(L)-CAT gene in pPL55B and 5′ end-labeled at *Bam*HI. Lanes: −, no extract; +, incubation with 2.5, 5, 10, and 20 μl of affinity-purified ProtA-Zta fusion protein.

sites, which are referred to herein as the ZRE3 (TGTG1) and ZRE4 (TGTG2) elements (see Fig. 10). The easier detection of the ZRE3 and ZRE4 sites with the B-Zta protein appeared to reflect the higher concentration of the specific ZRE-binding polypeptide in this preparation compared with that in the IgG-purified ProtA-Zta extract, rather than any major differences in specificity (data not shown).

Zta also binds efficiently to canonical AP-1 sites but not to point mutant forms of consensus core ZRE or TRE oligonucleotides. To confirm that the predicted core consensus sequence TGTGCAA at the center of the strong footprinted region on the enhancer fragment represents part of the actual recognition sequence for binding by the Zta fusion protein, we synthesized two new 30-mer oligonucleotide pairs, one including 20 bp from the wild-type binding site sequence TGTGCAA (ZRE5) and the other containing exactly the same sequences except for a 2-bp transposition point mutation TGGTCAA within the core consensus (ΔZRE5). The former bound to the bacterial Zta protein in a gel mobility retardation assay with a 5- to 10-fold higher affinity than did the 30-mer TGTG1 oligonucleotide, whereas binding to the point mutation was undetectable (Fig. 7a). All of the ZRE core motifs identified here diverge by only one or two bases from canonical AP-1 binding sites (TGAGTCA or TGAC TAA) that are found in the simian virus 40 enhancer, the metallothionine promoter, and the collagenase promoter and are known to be targets for the transcription factors c-Jun and c-Fos (2, 3, 40, 41). Interestingly, an isolated 18-bp fragment containing a known functional AP-1 site (TGA GTCA) from within the collagenase TRE bound efficiently to Zta also, although with slightly lower affinity than did the ZRE5 binding site (Fig. 7a). An otherwise identical DNA fragment containing a point mutation that abolishes AP-1 binding (ΔTRE, GGAGTCA) failed to bind to the Zta fusion protein. Weak binding was also detected with a 30-bp oligonucleotide containing an AP-1-related sequence motif (RR2) from the herpes simplex virus type 2 ICP10 promoter (Fig. 3c and 7a), but no binding was observed with our cyclic AMP response element consensus oligonucleotide from cytomegalovirus (7).

Transactivation of the collagenase TRE by *zta*. To assess the potential role of AP-1 sites in the mediation of *zta* responses, a TRE/TK-CAT plasmid containing five copies of the collagenase 18-bp AP-1 site (3) was also tested in cotransfection assays for its ability to respond to SV-Z transactivation. The level of CAT expression was found to be increased an average of 45-fold over basal activity in IB4 lymphoblastoid cells in the presence of *zta* effector DNA (Fig. 8; Table 1, experiments 1, 2, and 3). In the same experiments, the DSL enhancer construction ENH/A10-CAT functioned as a powerful *zta*-responsive promoter in IB4 cells, giving an average of 250-fold activation, whereas a control ΔTRE/TK-CAT construction containing a single copy of the mutant AP-1 binding site was stimulated only 4-fold (Fig. 8; Table 1). In Vero cells, TRE/TK-CAT gave high basal activity, which was usually decreased by cotransfection with the SV-Z effector plasmid, whereas the leftward DSL minimal promoter MIN(L)-CAT construction was ac-

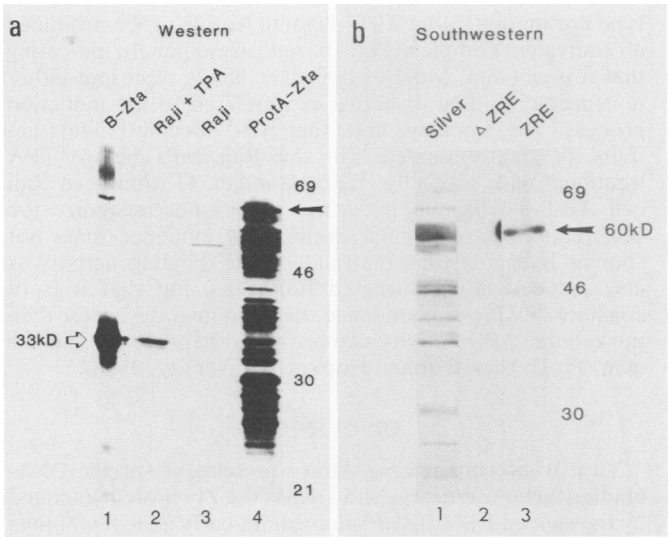

FIG. 5. Detection of bacterially expressed Zta proteins with specific antibody and ZRE DNA probes. (a) Western immunoblot assay showing Zta and Protein A-related polypeptides in partially purified preparations of B-Zta and ProtA-Zta or in induced Raji cell nuclear extracts. The probe used was the anti-Zta rabbit polyclonal antiserum directed against a Zta-specific peptide. Lanes: 1, 500 mM NaCl heparin agarose fraction from an *E. coli* B-Zta extract; 2, crude nuclear extract from 48-h TPA-induced Raji cells; 3, equivalent nuclear extract from untreated Raji cells; 4, IgG column affinity-purified fraction from an *E. coli* ProtA-Zta extract. (b) Southwestern blot assay identifying the intact 60-kDa polypeptide as the major ZRE5-binding activity within the IgG affinity-purified fraction from an ProtA-Zta bacterial extract. Lanes: 1, Silver-stained polyacrylamide gel electrophoresis profile of polypeptides present in the eluate from an IgG column; 2 and 3, autoradiographic images of the results of incubating filter-bound polypeptides from a parallel sample with ^{32}P-labeled oligonucleotide probes. The positions of rainbow reference proteins are indicated.

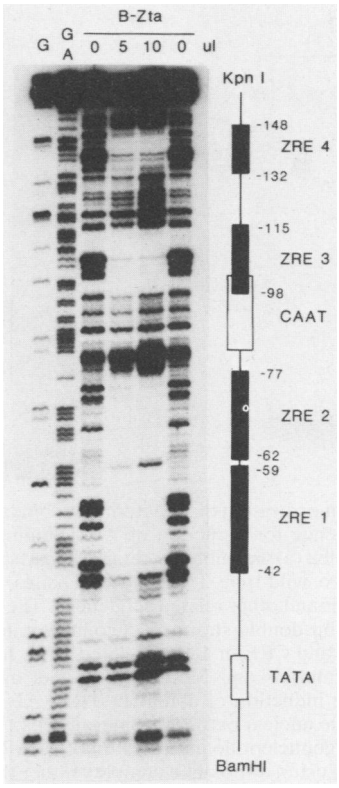

FIG. 6. Demonstration of the presence of four distinct Zta-binding sites in the minimal promoter region. The autoradiograph shows a partial DNase I footprint analysis of the minimal response region in the leftward DSL promoter with a nonfusion version of the bacterial-expressed Zta protein (B-Zta). The 200-bp *Bam*HI-to-*Kpn*I fragment from the MIN(L)-CAT gene was 5′ end labelled at *Bam*HI and incubated with 0, 5, or 10 μl of a heparin agarose fraction of partially purified B-Zta protein. G and G+A chemical sequencing tracks are shown in the two lefthand lanes.

tivated 23-fold (Table 1, experiment 4). Note that neither of the parent TK-CAT and A10-CAT constructions responds significantly to Zta in either Vero or IB4 cells (26, 42). Cotransfection with similar input DNA amounts of SV–c-Jun or SV-JunB plasmids gave no activation of either the ZRE or TRE targets in IB4 or Vero cells, and neither did cotransfection with SV–c-Fos or a mixture of SV–c-Fos plus SV–c-Jun (Fig. 8; Table 1). There were also no synergistic effects of cotransfection of SV–c-Jun or SV–c-Fos with SV-Z (data not shown).

Single-copy oligonucleotides containing ZRE sites convey Zta responsiveness. More direct evidence that the Zta-binding sites do indeed represent functionally significant ZRE came from experiments in which the ZRE5, ΔZRE5, ZRE3 (TGTG1), and ZRE6 (TGTG3) oligonucleotides were placed upstream of the unresponsive TK-CAT and A10-CAT reporter genes. Cotransfection of SV-Z DNA with TK-CAT containing either a single copy of the wild-type ZRE5 site or a dimer of the ZRE3 (TGTG1) site gave between 7- and 14-fold activation in Vero cells, compared with a slight shutoff of the somewhat higher basal activity in the ΔZRE5 version (Table 2, experiment 1). Similarly, the addition of single copies of the ZRE6 (TGTG3) oligonucleotide in either orientation conferred between 12- and 20-fold responsiveness to A10-CAT, compared with a 2-fold effect on the parent construction in IB4 cells (Table 2, experiment 2). Note that this represents only a small fraction of the effect produced with the full 256-bp enhancer region in a parallel assay.

Induction of AP-1-binding activity in TPA-induced Raji cell extracts. The ability of Zta to transactivate TRE/TK-CAT in IB4 cells but not in Vero cells implied that cellular AP-1-binding activity (presumably including c-Jun/c-Fos levels) may be low in the uninduced lymphoblasts. Similarly, the use of TPA to induce lytic cycle infection in EBV-carrying B-cell lines suggested the possible involvement of AP-1-binding activity in the activation of viral promoters. Nuclear extracts were prepared from latently infected Raji cells, both before and 48 h after treatment with TPA and sodium butyrate. The effectiveness of the induction was confirmed by Western blotting with the Zta-specific antiserum, which detected the 33-kDa Zta polypeptide in unfractionated crude extracts of the TPA-treated cells but not in the control untreated cell extracts (Fig. 5a, lanes 2 and 3).

The levels of binding to wild-type and mutant forms of both the TRE and ZRE sites were then examined by gel mobility retardation assays. An 18-bp TRE DNA probe isolated from the TRE/TK-CAT plasmid gave one very prominent new DNA complex with the TPA-induced extract but not with the uninduced Raji cell extract (Fig. 7b, left-hand panel). This binding activity was specifically related to the AP-1 consensus site as judged by its absence with the mutant ΔTRE probe. However, neither the wild-

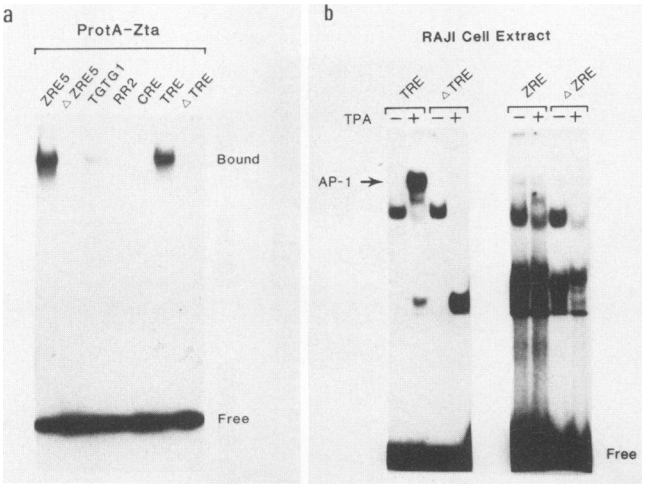

FIG. 7. Demonstration that bacterial Zta binds to canonical AP-1 sites and evidence for induction of AP-1-binding activity in TPA-treated Raji cells. (a) Gel mobility retardation assay showing binding of ProtA-Zta to wild-type TRE and ZRE sites but not to mutant ΔTRE or ΔZRE and other related sequences. The ^{32}P-labeled probes used were 30-bp double-stranded oligonucleotides ZRE5, ΔZRE5, TGTG1, RR2, and CRE or 18-bp isolated DNA fragments TRE and ΔTRE (see Materials and Methods). (b) Gel mobility retardation assay showing induction of abundant TRE (AP-1)-specific binding activity in crude nuclear extracts from Raji cells. The lack of binding to the ZRE oligonucleotide indicates that this AP-1-binding activity is likely to be a c-Jun–c-Fos-like complex rather than Zta. Lanes: −, extract from untreated cells; +, extract from cells after treatment with TPA and sodium butyrate for 48 h.

type nor mutant 30-bp ZRE oligonucleotide probe produced an equivalent complex (Fig. 7b, right-hand panel), indicating that it is not Zta. Most of the other bands represent either nonspecific binding or activities unrelated to the induction process. We conclude, first, that AP-1-specific binding activity is greatly depleted in the Raji cells before TPA treatment and, secondly, that the major TPA-induced Raji cell AP-1-specific binding activity does not recognize the ZRE consensus elements. Preliminary evidence (data not shown) indicates that Zta-related ZRE-binding activity is also induced in TPA-treated Raji cells, but that it is of considerably lower abundance than and migrates faster than the cellular AP-1 activity demonstrated here (P. M. Lieberman, Ph.D. thesis, Johns Hopkins University, 1988).

DISCUSSION

Viral transcriptional regulatory proteins as specific DNA-binding factors. We have shown that the Zta protein encoded by the spliced EBV BZLF1 immediate-early gene (*zta*) binds specifically to a family of consensus ZRE recognition sites and transactivates genes containing these ZRE sites in a cell type- and flanking sequence-dependent manner. Unlike the nuclear oncogenes of retroviruses, transcriptional regulatory proteins encoded by DNA viruses have not previously been shown to have obvious evolutionary homology with cellular transcription factors. Furthermore, the E2 protein from papillomavirus (1, 48, 61) and the EBNA-1 protein from EBV (55, 57) are the only other known DNA virus-encoded transcriptional regulatory proteins whose activator functions are thought to involve direct sequence-specific DNA binding. In contrast, the E1A protein from adenovirus is an indirect transactivator that apparently interacts with or modifies a variety of cellular DNA-binding transcription

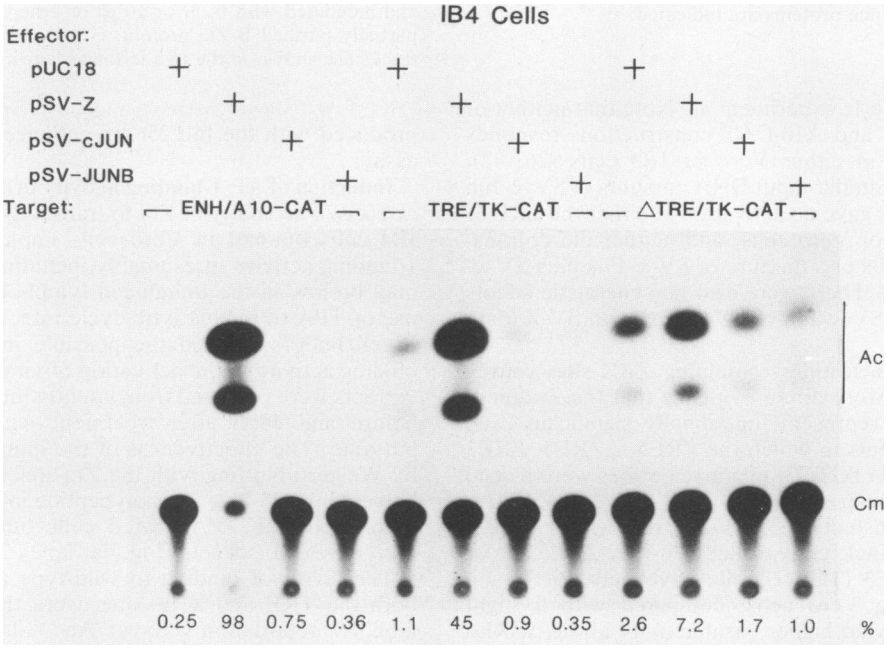

FIG. 8. Cotransfection with *zta* activates expression from a heterologous target promoter containing added TRE elements. (a) The autoradiograph shows transient CAT assays demonstrating *zta*-stimulated expression of a reporter gene containing a multicopy version of the wild-type collagenase TRE sequence but not that of a single-copy point-mutant control in IB4 cells. Samples of ENH/A10-CAT, 5×TRE/TK-CAT, or ΔTRE/TK-CAT target plasmid DNAs (2 μg each) were cotransfected with 2 μg of pUC18, SV-Z, SV–c-Jun or SV-JunB effector plasmid DNAs. Values for percent conversion of [^{14}C]chloramphenicol (Cm) to 1′- and 3′-acetylchloramphenicol (Ac) are given below the lanes.

TABLE 1. Zta responsiveness of collagenase TRE elements in cotransfection experiments

Expt and target gene	CAT activity, % conversion (fold induction[a])					
	Basal	Effector genes				
		SV-Z	SV-c-Jun	SV-JunB	SV-c-Fos	SV-c-Jun plus SV-c-Fos
Expt 1 (IB4)						
TRE/TK-CAT	1.1	45 (42)	0.9 (0.9)	0.4 (0.4)		
ΔTRE/TK-CAT	2.6	7.2 (2.7)	1.7 (0.7)	1.0 (0.4)		
ENH/A10-CAT	0.25	98 (390)	0.7 (2.7)	0.4 (1.7)		
Expt 2 (IB4)						
TRE/TK-CAT	0.6	12 (20)	0.5 (0.8)		0.4 (0.7)	0.3 (0.5)
ΔTRE/TK-CAT	0.5	2.4 (4.8)	0.5 (1.0)		0.25 (0.5)	0.4 (0.8)
ENH/A10-CAT	0.25	55 (220)	0.5 (2.0)		0.6 (2.4)	0.5 (2.0)
Expt 3 (IB4)						
TRE/TK-CAT	0.21	15 (73)	0.16 (0.8)			
ENH/A10-CAT	0.22	34 (153)	0.34 (1.6)		0.22 (1.0)	0.16 (0.7)
Expt 4 (Vero)						
TRE/TK-CAT	15	11.2 (0.8)	12.5 (0.8)	16.6 (1.1)		
ΔTRE/TK-CAT	10.4	13.0 (1.2)	20 (1.9)	9.7 (0.9)		
TK-CAT	1.0	2.2 (2.2)	0.7 (0.7)	0.5 (0.5)		
MIN(L)-CAT	0.8	28 (34)	0.5 (0.7)	0.3 (0.4)		

[a] Ratio of percent conversion in the presence of 2 μg of effector DNA compared with basal expression in the presence of 2 μg of pBR322 DNA.

factors to increase the frequency or stability of template recognition by RNA polymerase (28, 35, 44, 67). Similarly, the virion component of herpes simplex virus (VF65, VP16, or αTIF), although acting only on specific targets, also stimulates transcription of the herpes simplex virus immediate-early genes indirectly by formation of a complex with the cellular transcription factor OTF-1(NFIII) and the cognate target DNA sequence (4, 22, 50, 53).

Conservation of a basic amino acid DNA-binding domain in the Jun-Fos-Zta protein family. Intriguingly, the highly basic second exon (36 amino acids) of the Zta protein (5) has extensive amino acid similarity to the third exon (35 amino acids) of the c-Fos proto-oncogene (19). A conserved homologous basic region is also found in the c-Jun–JunB–Fra-1–CREB and C/EBP family of mammalian nuclear DNA-binding proteins and in the GCN4 yeast transcription factor (Fig. 9). This domain has been shown to be essential for DNA binding (62, 66), along with an adjacent conserved "leucine zipper" domain that is involved in dimerization (34, 37, 60).

TABLE 2. Addition of Zta-binding sites to heterologous promoters confers Zta responsiveness

Expt and target gene[a]	CAT activity		
	% Conversion		Fold increase
	Basal[b]	With SV-Z	
Expt 1 (Vero cells)			
ZRE5 (+)/TK-CAT	1.2	8.4	7.0
ΔZRE5 (+)/TK-CAT	3.0	1.9	0.6
2x(ZRE3)(+)/TK-CAT	0.6	8.5	14
TK-CAT	1.3	0.8	0.7
Expt 2 (IB4 cells)			
ZRE6(+)/A10-CAT	0.2	4.2	21
ZRE6(−)/A10-CAT	0.1	1.2	12
A10-CAT	0.1	0.2	2.0
ENH/A10-CAT	1.5	98	>65

[a] +, Forward orientation; −, backward orientation.
[b] Cotransfection of 2 μg of target DNA with 2 μg of pBR322 DNA (basal) or 2 μg of pPL17 DNA (SV-Z).

The c-Jun, JunB, Fra-1, and GCN4 proteins all bind specifically to consensus AP-1 target DNA sequences (TG AGTCA, TGACTCA, or TGACTAA), whereas the CREB protein (27) binds preferentially to the related consensus cyclic AMP response element (TGACGTCA). Similarly, the recognition sites for C/EBP include the somewhat related sequence TGTGGAA(AG) found in the simian virus 40 and murine sarcoma virus enhancers (31, 36). In contrast, the intact c-Fos protein does not form stable dimers and is believed to bind to AP-1 sites and to activate TRE-containing promoters by forming heterodimers with the c-Jun or Fra-1 proteins (11, 24, 49, 54, 59). All four different core Zta-binding site sequence motifs in the bidirectional DSL promoter region of EBV (e.g., the high-affinity TGTGCAA, TG TGTAA, and TGAGCAA sites and the lower-affinity TGTG TCA sites) are diverged significantly from the recognized AP-1 concensus site, although the EBV Zta fusion protein also recognizes the collagenase TRE AP-1 site (TGAGTCA) with relatively high affinity. In contrast, our experiments with both the major TPA-induced Raji cell AP-1-binding activity (Fig. 7b) and with in vitro-translated c-Jun and c-Fos (Y.-N. Chang et al., unpublished data) indicate that the TG TGCAA (ZRE5) site does not serve as a recognition site for cellular AP-1 proteins.

The various different DNA-binding properties of these factors suggest that amino acid differences within the conserved basic domains and perhaps the association of subunits into heterodimers compared with homodimers may dictate DNA-binding site specificity and stability. Note that the unspliced genomic BZLF1 fusion protein (containing exon-1 amino acids 1 to 167) did not have DNA-binding properties, implying that the 78-amino-acid spliced COOH-terminal domain of Zta (codons 168 to 245) is required for its DNA-binding properties, which parallels the findings for the

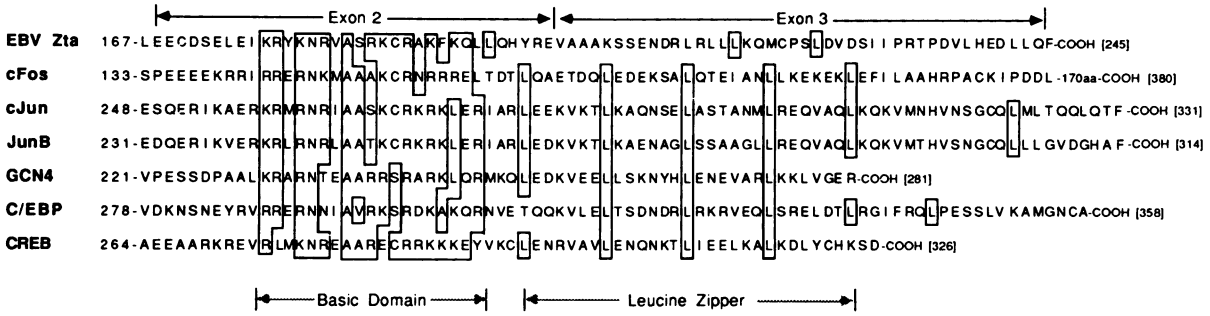

FIG. 9. Amino acid similarity between exons 2 and 3 of the EBV Zta transactivator protein and the COOH-terminal DNA-binding domains of members of the Fos-Jun-GCN4 Family of transcription factors. The conserved consensus residues in the highly basic domain and all leucine residues potentially involved in the adjacent leucine zipper domain are highlighted. Amino acid sequence data are derived from the following sources: Zta (5); human c-Fos (65); human c-Jun (6); mouse JunB (58); *Saccharomyces cerevisiae* GCN4 (29); rat C/EBP (36); human cyclic AMP-binding protein (27).

GCN4, c-Jun, and C/EBP proteins. However, the potential coiled-coil alpha-helical dimerization domain in the adjacent third exon of Zta is significantly diverged from the canonical leucine zipper pattern (Fig. 9), and there is no significant amino acid homology between the Zta protein and c-Fos or c-Jun outside of the small second exon. Structurally, Zta more closely resembles c-Jun than c-Fos, because both Zta and c-Jun lack the extended COOH-terminal region of c-Fos. Furthermore, by using in vitro translation and cross-linking studies, we have found that, unlike c-Fos, Zta forms a stable homodimer in both the bound and unbound states (Chang et al., unpublished data).

Importance of ZRE-binding site context. Although single ZRE sites have been shown here to be targets for Zta responses in transient assays, the particular arrangement of multiple ZRE sites relative to adjacent consensus sites in the divergent DSL promoter region (Fig. 10) strongly suggests that other protein-DNA binding interactions may contribute to the transcriptional induction mediated by the Zta protein. In the leftward DSL promoter, deletion studies have shown that the AACCAAT box sequence at position −95 delineates the 5′ border of the minimal response region in Vero cells and that the 3′ border includes the GATAAA element at position −30 (Lieberman, Ph.D. thesis). The affinity-purified Zta protein binds to two high-affinity sites (ZRE1 and ZRE2) centered at positions −52 and −70, encompassing a 35-bp domain between the TATA and the CAAT box elements as well as to two lower-affinity sites (ZRE3 and ZRE4) centered at positions −106 and −139. Thus the entire region contains multiple adjacent sites for binding by transcription factors that could potentially interact with one another.

In contrast, the ZRE5- and ZRE6-binding sites lie in a position- and orientation-independent upstream enhancer domain (positions −643 to −900) that responds strongly to *zta* only in EBV-transformed lymphoblasts (8, 15, 42). Part of the explanation for both the cell-type specificity and the enhancerlike properties of this region may be related to an adjacent cellular factor binding site, which overlaps with ZRE5 at positions −711 to −694 in the DSL region (Lieberman, Ph.D. thesis). Interestingly, this *Pvu*II factor recogni-

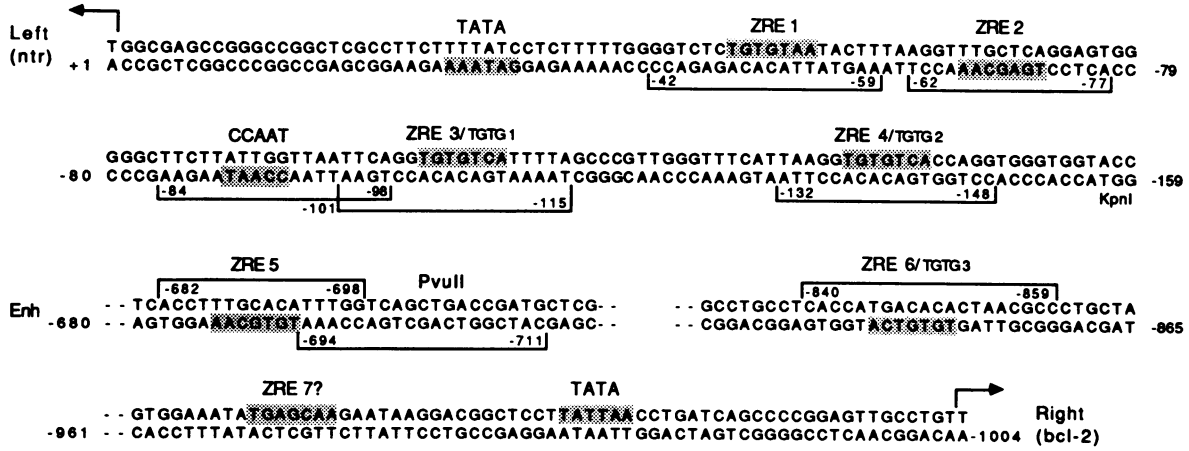

FIG. 10. Overall arrangement of ZRE sites and cellular transcription factor binding sites within the DSL divergent promoter. The core consensus sequences and the orientation and position of various factor-binding sites within relevant portions of the 1,004-bp intergenic region are highlighted. The DNase I-protected regions for ZRE sites 1, 2, 3, 4, 5, and 6 in the presence of the ProtA-Zta or B-Zta fusion proteins are derived from footprinting studies shown in Fig. 4 and 6. Boundary positions are given relative to the leftward mRNA start site. Additional evidence for binding to ZRE sites 3, 5, and 6 also comes from the oligonucleotide binding studies shown in Fig. 3 and 7. Binding to site 7 is predicted based on sequence homology to ZRE site 2. Evidence for cellular factors that bind to the *Pvu*II site, the CAAT site, and each of the three TGTG (GGTGTGTCA) elements is presented elsewhere (Lieberman, Ph.D. thesis). Note that the *Pvu*II factor-binding site partially overlaps with ZRE site 5 and that ZRE sites 3, 4, and 6 also correspond to TGTG factor-binding sites 1, 2, and 3, respectively.

tion site (GGTCAGCTGACC) closely resembles the consensus estrogen receptor-binding site sequence GGTCANNN TGACC (18, 51). The DSL enhancer region also contains response signals for another EBV immediate-early transactivator, R or Rta, which is the product of the BRLF1 (*rta*) gene (9, 26). The Rta gene product acts synergistically with Zta to activate the enhancer in EBV-negative lymphoblastoid cells (15).

AP-1-binding activity in EBV-immortalized cells. The fact that the TRE/TK-CAT gene gave high constitutive expression in Vero cells but not in IB4 cells appears to correlate with the lack of AP-1-specific DNA-binding activity detectable in the lymphoblastoid cells before TPA treatment (and with the presumed constitutive expression of functional c-Jun- or c-Fos-like proteins in Vero cells). This result also appears to account in part for the ability of the Zta protein expressed from the SV-Z plasmid to activate the TRE/TK-CAT target gene in cotransfection assays in IB4 cells. Nevertheless, we were unable to stimulate CAT expression from either the DSL enhancer or TRE/TK-CAT targets in IB4 cells by cotransfection with SV–c-Jun or SV–c-Fos or a combination of both. These latter effector plasmids have been reported to activate TRE/TK-CAT in F9 mouse teratocarcinoma stem cell lines, which are also depleted in basal AP-1-binding activity and have low levels of endogeneous c-Jun and/or c-Fos (11, 59).

The low basal levels of AP-1 DNA-binding activity in EBV-immortalized B lymphoblasts may be a key factor that helps to maintain the repressed latent state of the EBV genome. Clearly, *zta* can substitute for TPA in activation of the EBV lytic cycle, and expression of the Zta protein is itself induced by TPA treatment, but we do not know whether Zta is an essential intermediate during TPA activation. The results shown here indicate that a major AP-1-binding activity is induced in Raji cells, but that this does not bind to the consensus ZRE5 target sequences. Furthermore, since maximal stimulation of the synthesis of Zta protein and *ntr* mRNA in Raji cells takes up to 48 h, and since the induction of endogenous *zta* mRNA by TPA requires new protein synthesis (39), it seems likely that induction of the Zta protein itself involves a complex differentiation pathway rather than direct transient activation of AP-1-binding activity. Furthermore, neither the DSL target promoter nor the *zta* promoter region itself contain canonical AP-1 motifs. Interestingly, the promoters for two other EBV target genes (BMRF1 and BMLF1), which we and others have shown to respond to *zta* transactivation, do possess standard canonical AP-1 (TGAGTCA) sites at upstream positions (−70 and −100), which raises the possibility of *zta*-independent stimulation of some genes after TPA treatment. Nevertheless, both of these other promoters (one of which drives transcription of the gene encoding the nonspecific Mta transactivator protein) also possess multiple consensus ZRE (TGTGCAA-like) sites and TGTG (GGTGTGTCA-like) sites closely interspersed among CAAT box and TATA box elements.

Possible modification of a cellular transcriptional pathway by the virus. The most fascinating conclusion from our studies and those of Farrell et al. (19) is that EBV utilizes a mechanism for lytic-cycle gene regulation in which it appears to have subverted components of a normal cellular transcription pathway to give specific activation of viral genes. We speculate that this has been accomplished by (i) capturing a c-*jun*-related cellular activator gene or at least an exon from one; (ii) modifying or relaxing both the target specificity for this protein and the response signals within its own promoters to include a much wider variety of diverged AP-1-like sequence motifs; and (iii) discarding the usual functional requirement for formation of c-Jun/c-Fos heterodimer complexes, such that Zta can now perhaps act directly as a stable homodimer in the absence of any cellular c-Fos activity. Note that these features of EBV lytic cycle gene regulation are quite distinct from those described for alpha and beta herpesviruses (e.g., herpes simplex virus and cytomegalovirus) and that there are no known homologous counterparts of *zta* (or *rta*) in any other human herpesvirus. Therefore, these genes may represent relatively recent evolutionary additions to the primordial gamma herpesvirus genome. Furthermore, in contrast to other apparently recently acquired herpesvirus subgroup-specific genes (e.g., human lymphocyte antigen class I and β-adrenergic receptor-like genes in human cytomegalovirus and dihydrofolate reductase and thymidylate synthetase in herpesvirus saimiri), the *zta* gene in EBV may have retained at least part of its original intron-exon structure.

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